RABIES VIRUS IN ARCTIC FOX (*VULPES LAGOPUS*): A STUDY OF PANTROPIC DISTRIBUTION

By

Lori A. Gildehaus

RECOMMENDED:

Advisory Committee Co-Chair

Chair, Biology and Wildlife Department

APPROVED:

Dean, College of Natural Science and Mathematics

Dean of the Graduate School

Dec 6, 2010

Date

RABIES VIRUS IN ARCTIC FOX (*VULPES LAGOPUS*): A STUDY OF PANTROPIC DISTRIBUTION

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Lori A. Gildehaus, B.S.

Fairbanks, Alaska

December 2010

Abstract

Rabies is endemic in Arctic foxes, in Alaska and other Arctic regions and cold temperatures may preserve the virus in Arctic climates in infected animal carcasses. These frozen carcasses may provide a source of infection throughout winters and thereby propagate the rabies virus within animal populations in the Arctic.

It was hypothesized that rabies virus antigen is present in the soft tissues of naturally infected Arctic foxes, *Vulpes lagopus*. Using a direct rapid immunohistochemistry test (DRIT) and a fluorescent antibody test (FAT), thirteen organ tissues from twelve naturally infected and three experimentally infected Arctic foxes were tested. All tissues, except testes, tested positive for rabies virus antigen by the DRIT, the FAT, or both in at least one fox. Although the DRIT detected rabies virus antigen in non-neuronal tissues, it did not detect antigen in as many non-neuronal tissues as the FAT. Spleen and stomach tissues had the highest rate of rabies virus detection by the FAT and using a combination of non-neuronal tissues would be the best substitute for brain if brain were unavailable.

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General Introduction

Currently, rabies persists primarily as a disease of wildlife in the United States and developed nations (Krebs et al. 2001, 2002), and wildlife accounts for the majority of human rabies exposure cases in the United States (Hankins and Rosekrans 2004). Approximately 20,000 to 40,000 people are treated for rabies exposure annually in the United States (Wilde 1997). In recent times, more than 90% of rabies cases that were reported each year to the Center for Disease Control (CDC) from the United States and Puerto Rico were in wildlife (Blanton et al. 2010). The most important vectors are skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), bats (*Chiroptera*), and red foxes (*Vulpes vulpes*) (Mrak and Young 1994, Jackson 2000). In the contiguous United States, these animals are reservoirs of the virus. In the Arctic and subarctic, the Arctic fox (*Vulpes lagopus*) is the most significant reservoir (Cowan 1949, Crandell 1991, Krebs et al. 2001) and rabies is enzootic in Arctic fox populations in Alaska. Rabies is also endemic in other Arctic fox populations, such as those in Svalbard, Greenland, and Canada (Secord et al. 1980, Prestrud et al. 1992, Mansfield et al. 2006).

The dynamics of rabies virus in Arctic foxes has led to speculation that the virus may be transmitted via a non-bite route. If rabies virus is present in the organ tissues of naturally infected Arctic foxes, then this may be a source of rabies infection throughout winters in the Arctic. Although no studies have assessed viral infectivity of rabies-infected carcasses in the Arctic, there is evidence supporting the theory that cold temperatures may help preserve the virus (Hanlon et al. 2007, Krauss 2003, Crandell 1991). Most rabies-related deaths in the Arctic occur in winter, so it is

conceivable that the virus remains viable after the death of an infected fox because of freezing temperatures, and that scavenging animals can contract the virus. It has been proposed that scavenging of rabid fox carcasses may propagate the virus in animal populations (Ballard et al. 2001). Furthermore, scavenging of Arctic fox carcasses in northern Alaska is quite common (Pamperin 2008, Dr. E. Follmann, pers. comm.). Transmission of rabies virus could occur by abrasion of mucous membranes by bone shards and frozen tissues to scavenging animals.

Chapter 1 Rabies Virus in Arctic fox (*Vulpes lagopus*): A Study of Pantropic Distribution[1]

Background

Rabies in Wildlife

The rabies virus, a member of the genus Lyssavirus, causes a neurotropic disease of warm-blooded animals resulting in encephalitis and, eventually, death. The name rabies has its root in the Latin verb "rabere", meaning "rage". It is an extremely old disease, known since the start of recorded history. Historically, rabies in the Arctic has been a disease of wild and domestic animals, with epizootics occurring in dogs and foxes (Elton 1931). Today, rabies still remains a very serious public health threat, claiming approximately 55,000 human lives worldwide every year (Wilde et al. 2008) although the true estimate may be as high as 70,000 (Hankins and Rosekrans 2004). Most human rabies cases occur in developing nations, primarily because they lack resources to implement vaccination programs in domestic animals, and, thus, occur due to interaction with rabid domesticated animals, predominantly dogs (Kasempimolporn et al. 2004, Baer 2007, Jackson 2007a). Due to the pioneering efforts of vaccination programs in the 1940's, the number of infected domestic animals has been drastically reduced in the United States (Mrak and Young 1994, Finnegan et

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[1] Gildehaus, L., E. H. Follmann, J. Runstadler, L. Dehn, and G. Happ. 2010. Rabies virus in Arctic fox (*Vulpes lagopus*): a study of pantropic distribution. Prepared for Journal of Wildlife Diseases.

al. 2002). However, rabies endures in wildlife populations (Finnegan et al. 2002, Baer 2007).

Spillovers can occur between species, especially during epizootics when there is a high rate of viral transmission. According to the State of Alaska Epidemiology Bulletins (No. 16 1978, No. 5 2000, No. 26 2002, No. 20 2006), caribou (*Rangifer tarandus*), bats (*Myotis lucifugus* and *Myotis keenii*), river otters (*Lutra canadensis*), wolves (*Canis lupus*), coyotes (*Canis latrans*), and reindeer (*Rangifer tarandus f. dom.*) have tested positive for rabies virus. Canada has also reported rabies infection in seven black bears (*Ursus americanus*) and one polar bear (*Ursus maritimus*) (Taylor et al. 1991, State of Alaska Epidemiology Bulletin No. 26 2002). While spillovers of rabies into the marine ecosystem appear to be rare, only one ringed seal (*Pusa hispida*) was identified as rabies-positive in Norway (Ødegård and Krogsrud 1981), the potential of increased contact between bears, foxes, and seals due to climate change is noteworthy. Spillovers from wildlife populations into domestic animal populations increase potential human exposure to rabies virus (State of Alaska Epidemiology Bulletin No. 20 1997, Blanton et al. 2010). Human exposure may occur when hunters and trappers handle infected fox carcasses or through a bite from infected wildlife (State of Alaska Epidemiology Bulletin No. 20 1997).

Rabies Epizootics

Rabies epizootics were documented between foxes and dogs in 1768 when rabies was first recorded in North America (Baer 2007). Currently, rabies is enzootic

throughout the Arctic and epizootics exhibit a cyclical pattern (Prestrud et al. 1992, Mansfield et al. 2006). In Alaska, epizootics occur every 3-4 years (Elton 1931, Chesemore 1975, Figure 1), most often along the north and west coasts. Outbreaks are more likely to occur during winter months following viral transmission in the fall (Elton 1931, Kantorovich 1964, Ritter 1981, Crandell 1991), with up to 75% of the population becoming infected (Elton 1931, Kantorovich 1964, Mørk and Prestrud 2004). The cyclic nature of rabies outbreaks makes eradication difficult. During the years between epizootics a low percentage of animals in the population may still harbor the virus (Kantorovich 1964), but the numbers of outbreaks are fewer.

Rabies epizootics in Arctic foxes may also be dependent on food availability. The primary prey of the Arctic fox is the brown lemming (*Lemmus sibiricus*) (Elton 1931), but foxes will also feed on other rodents, birds, and carrion (Chesemore 1975). When food is abundant and the population density can increase, this may result in an increase in rabies prevalence (Mørk and Prestrud 2004, Holmala and Kauhala 2006) and outbreaks of rabies (Elton 1931, Kantorovich 1964, Ritter 1981), presumably because of increased contact between foxes. Furthermore, factors such as increased population density and decreased food availability may induce stress, which may also increase the animal's susceptibility to rabies or disease in general (Steele 1973, Lafferty and Holt 2003). Animals with weakened immune systems may be more susceptible to virus infection (Elton 1931, Mateo et al. 2006). This is in agreement with other virus studies that have suggested that disease may manifest itself in populations when densities increase or when animals are malnourished (Trainer and

Knowlton 1968). Conversely, years of low prey abundance, when foxes experience food scarcity in the Arctic winter, may also induce stress and thereby increase their susceptibility to the rabies virus (Elton 1931).

Viral Shedding

Rabies virus was isolated from salivary glands of Arctic foxes during years of increased population size or during migration, but not during years when the population was markedly small or during periods when foxes were non-migratory (Kantorovich 1964). This indicates that viral shedding occurs during periods of increases in population density, presumably when animals may be stressed and in search of food. It is unknown how long viral excretion persists, but has been shown to occur for up to 305 days after inoculation in experimentally infected dogs (Fekadu et al. 1981).

Arctic Fox Population Cycle

Arctic fox populations peak during early summer with the emergence of young from the dens. Although viral transmission is presumably low during this time because there is little contact among animals between adjacent territories, a study demonstrated that rabies virus could be passed transplacentally (Martell et al. 1973). However, maternal antibodies against rabies can also be transferred from mother to offspring (Muller et al. 2002). During fall and early winter, the territories break down as foxes disperse and migrate in search of food (Pamperin et al. 2008, Dr. E.

Follmann, pers. comm.). As fox movement and contact increases, viral transmission and frequency of infection also increases (Ritter 1981). In late winter and early spring, female and male foxes form monogamous pairs and occupy underground dens to breed thereby decreasing transmission likelihood (Chesemore 1975).

Non-bite Transmission

Transmission of rabies virus generally occurs via a bite from the infected animal that exposes subcutaneous tissue, nerve endings and muscle to infected saliva, but infection can occur through ingestion of rabies-infected tissue, although it is largely unknown how the virus gains entry into the nerves. A study by Soave (1966) demonstrated that two species of mice (*Calomys musculinus* and *Mus musculus*) became infected with rabies virus when they ingested naturally infected tissues of vampire bats (*Desmodus rotundus*), bovines, and dogs. The study also revealed that, while several mice died of rabies infection, numerous mice seroconverted after ingesting the rabid tissue (Soave 1966). Additionally, a study performed on wild birds found that predatory and non-predatory birds had antibodies against rabies antigen (Gough and Jorgenson 1976). Interestingly, the non-predatory birds that tested positive for antibodies were primarily scavengers and were most likely exposed to the virus orally through infected carcasses (Gough and Jorgenson 1976). Oral exposure of avian scavengers to rabies virus is in agreement with studies suggesting that wildlife may acquire rabies orally through ingestion of frozen carcasses (Mørk and Prestrud 2004).

Incubation Period

The incubation period is generally considered the time from when infection occurs to when the animal becomes symptomatic (Nadin-Davis 2007). Long incubation periods may last days, weeks, months, or possibly years (Blancou 1988, Smith et al. 1991) with the virus sequestered in the muscle at the site of inoculation (Charlton et al. 1997). The variation in length of the incubation period may be due to virus travel distance to reach the CNS, and nerve density in the area (State of Alaska Epidemiology Bulletin No. 5 2000), but it is largely unknown if the virus has any other activity during the incubation period (Jackson 2007b). The virus gains entrance to the nervous system through neuromuscular junctions (Murphy et al. 1973, Watson et al. 1981). However, cells that do not express nicotinic acetylcholine receptors can also be infected by rabies virus, indicating that there are other routes the virus can use to enter the cell (Seganti et al. 1990). Once the virus gains access to peripheral nerves it moves via retrograde axonal transport within the nerves to the central nervous system.

Once the brain becomes infected, the victim becomes symptomatic as the virus replicates in neurons. The manifestation of symptoms appears to be the result of neuronal dysfunction rather than cell death, as cell death is often minimal with rabies infection (Rossiter and Jackson 2007). The virus then centrifugally spreads within the CNS by fast axonal transport to the eye, salivary glands, and systemic organs (Jackson et al. 1999, Jackson 2007b). As nasal epithelium and salivary gland

epithelium become infected the animal begins shedding the virus in oral secretions. When individuals begin shedding the virus, they are able to infect others and thereby propagate the virus within the population, and by spillovers into other animal populations.

To explore how rabies virus is transmitted within Arctic foxes and potentially within the Arctic, I conducted a study to assess pantropic distribution of rabies virus antigen within various organs of naturally and experimentally infected Arctic foxes. I hypothesize that rabies virus is present in organ tissues of infected Arctic foxes and that rabies infected fox carcasses may serve as a source of infection to foxes and other scavengers.

Introduction

Epizootics in Arctic Fox Populations

Arctic foxes are highly susceptible to rabies virus and seem to be natural hosts to the disease (Konovalov et al. 1965, Blancou 1988). Although Arctic foxes are susceptible to viral infection they may not be as susceptible to overt disease. In captive studies, some Arctic foxes have failed to develop disease when inoculated with high doses of rabies virus, despite testing negative for rabies antibodies prior to virus challenge (Follmann et al. 2004). Additionally, rabies neutralizing antibodies have been detected in naturally infected Arctic foxes indicating that some foxes exposed to the virus in the wild may naturally seroconvert (Ballard et al. 2001, Dr. E. Follmann, pers. comm.). In the wild foxes may appear healthy and remain

asymptomatic during infection (Elton 1931, Kantorovich 1964, Mørk and Prestrud 2004). Furthermore, Arctic foxes can survive prolonged incubation periods when infected with the virus, which may be a way for the virus to persist in enzootic populations (Jackson 2007b).

Virus Distribution and Transmission

Viral transmission of rabies virus can occur through an open cut as is evidenced by a survey by Follmann et al. (1994), who detected rabies antibodies in an Alaskan trapper who had not received previous vaccinations or pre- or post-exposure prophylaxis treatment and who did not report being bitten. Non-bite viral transmission is further supported by human-to-human transmission through organ transplants (Houff et al. 1979, Center for Disease Control 2004, Burton et al. 2005, Bronnert et al. 2007), and a postmortem study in humans that revealed rabies virus antigen in extraneural organs (Jackson et al. 1999). Numerous other studies in experimentally infected animals have also detected rabies virus antigen in organ tissues (Debbie and Trimarchi 1970, Murphy et al. 1973, Balachandran and Charlton 1994). These infections support the hypothesis that the rabies virus is present in organ tissues and can be transmitted through them.

In rare circumstances, non-neuronal transmission has been documented through infected blood or lymph (Dean et al. 1963) and through intact mucous membranes (Fischman and Wards 1968). This type of transmission may be more likely to occur in young animals that have underdeveloped immune systems or in animals that are

highly susceptible to the rabies virus infection (Dean et al. 1963). Furthermore, rabies virus RNA has been detected in cerebral spinal fluid, saliva, tears, and urine of infected humans (Hemachudha and Wacharapluesadee 2004), and in the urine of experimentally infected dogs (Sitprija et al. 2003).

Diagnostic Tests

To conduct this study, I used a direct rapid immunohistochemistry test (DRIT) and a fluorescent antibody test (FAT) to detect rabies virus antigen in organ tissues of naturally and experimentally infected Arctic foxes. The FAT is a highly sensitive test for rabies, comparable to virus isolation (Trimarchi and Nadin-Davis 2007), and is used globally as the primary diagnostic test to identify rabies infection in the brain (Rudd et al. 2005). Fluorescent antibody testing has been used in numerous studies to detect rabies virus antigen in non-neuronal tissue and determine tissue tropism (Debbie and Trimarchi 1970, Martell et al. 1973, Fekadu and Shaddock 1984, Balachandran and Charlton 1994). Fluorescent antibody testing is very reliable and all human rabies cases resulting from animal bites in North America have been accurately diagnosed (Trimarchi and Nadin-Davis 2007). The FAT uses a single fluorescently tagged antibody to bind to the antigen and detection is observed by green fluorescence (Figure 2).

Immunohistochemical testing has also proven very sensitive in detecting rabies viral antigen (Jogai et al. 2000). The direct rapid immunohistochemistry test (DRIT), a test recently developed by the CDC, has been shown to be as effective and accurate

at diagnosing rabies virus antigen in brain tissue as the FAT (Lembo et al. 2006). It uses a biotinylated primary antibody coupled with streptavidin horseradish peroxidase to produce a dark reddish brown staining of rabies virus antigen (Figure 2). The DRIT has proven to be a highly reliable test when used on neuronal tissue, compared to FAT (Lembo et al. 2006) and was, therefore, the choice for a secondary diagnostic test. Incubation of specimens with the necessary antibody cocktails used in the DRIT can be performed at room temperature and requires only a light microscope. FAT, in contrast, requires a fluorescent microscope and a humidity chamber. Thus, the DRIT can be performed in remote field locations with minimal laboratory equipment.

Objectives

Both immunohistochemistry and fluorescent antibody testing permit visualization of rabies antigen and provide rapid results. However, while the FAT requires costly equipment the DRIT requires only a light microscope. The relative ease of testing makes DRIT a potentially powerful tool in remote areas of Alaska and elsewhere in the Arctic, in particular if submissions of suspect rabies cases in rural communities cannot be tested in a timely manner (due to flight schedules and increment weather) and yet immediate results are needed.

Using the DRIT and FAT, the objectives of my study were to: 1) determine the presence of the rabies virus antigen in a variety of soft tissues of naturally and experimentally infected Arctic foxes, 2) establish if the DRIT and FAT can detect rabies virus antigen in non-neuronal tissue in Arctic foxes, and 3) investigate if a non-

neuronal tissue can be a suitable replacement for brain tissue in diagnosing rabies if the brain is unavailable for analysis.

Methodology

Sampling Procedure

Twelve naturally infected Arctic foxes were collected in 1994 and 1997 from Barrow, Alaska and surrounding enzootic regions and were tested for rabies virus antigen in soft organ tissues (Table 1). Brains of these animals tested positive for rabies virus via FAT at the Alaska State Virology Laboratory (ASVL), and carcasses were stored frozen at -20°C at the University of Alaska Fairbanks. The foxes were thawed at room temperature in small batches (2-4 foxes) until carcasses could be manipulated and samples taken, which was approximately after 16 hours. Tissue samples were taken from the following organs: bladder, esophagus, heart, kidney, large intestine, liver, lung, ovary, pancreas, semitendinosus muscle, small intestine, stomach, spleen, and testes. I chose to test primarily large organ tissues that would be easily accessible to a scavenging fox in the wild. Due to advanced decomposition of several animals it was either not possible to obtain tissue samples or only small tissue samples (approximately two grams) were collected to perform the DRIT or the FAT but not both (Tables 2). Tissues from three experimentally infected Arctic foxes were available from a separate investigation (Dr. E. Follmann, unpublished data) and were also included in this study (Table 1). The experimentally infected Arctic foxes were challenged with high titer rabies virus bilaterally into the masseter muscle. Foxes

EXP 10 and EXP 12 (challenged with 500,000 MLD_{50}) were euthanized after they became symptomatic, but were not yet moribund 13 and 20 days after infection, respectively. Fox EXP 349 (challenged with 50,000 MLD_{50}) died 18 days after infection. Tissue samples were taken and stored at -20°C until processed. For a negative control, tissue samples from an Arctic fox that tested negative for rabies virus by the Alaska State Virology Lab were also used and processed for the DRIT and FAT to confirm that non-specific binding did not occur in these tests (Table 2).

Frozen tissue samples were embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound, according to the manufacturer's instructions. Tissues were cut into 8um sections at -20°C onto specialized cellular adhesion slides (ERIE Scientific, Portsmouth, NH) using an I.E.C. Minotome cryostat. Tissue samples were tested in duplicate. Slides were kept frozen at -20°C until all tissues from a single fox were sectioned. DRIT and FAT were performed on the frozen tissue sections to determine presence or absence of rabies virus antigen within each tissue.

DRIT Protocol

The DRIT and FAT protocols were obtained from the CDC. Briefly, slides for the DRIT (Figure 3) were air dried and fixed in 10% phosphate buffered formalin for 10 minutes, dip-rinsed in a wash buffer of phosphate buffered saline with 1% Tween 80 (TPBS), then immersed in 3% hydrogen peroxide, and washed again in TPBS. Next slides were incubated for 10 minutes at room temperature in a humidity

chamber with a monoclonal antibody (MAb) cocktail (obtained from CDC), dip-rinsed in TPBS and incubated for 10 minutes at room temperature in a humidity chamber with streptavidin-peroxidase complex. Following incubation, slides were dip-rinsed again in TPBS. The slides were then incubated with a 3-amino-9-ethylcarbazole (AEC) peroxidase substrate for 10 minutes followed by a rinse in distilled water. Slides were then counterstained with hematoxylin diluted 1:2 with distilled water for two minutes and dip-rinsed in distilled water, and a cover slip was applied with a water soluble mounting media. Slides were read with a light microscope at 40X objective. Full DRIT protocol details can be found in Lembo et al. (2006).

FAT Protocol

For the FAT (Figure 4), slides were air dried at room temperature and incubated at -20°C in acetone for one hour. Slides were then incubated at 37°C in a humidity chamber with fluorescein isothiocyanate (FITC)-anti-rabies-monoclonal globulin with 0.00125% Evan's Blue counterstain. Slides were rinsed briefly with phosphate buffered saline (PBS) to remove any excess conjugate and were then immersed in PBS for three to five minutes. The slides were rinsed a second time for three to five minutes in new PBS, and allowed to briefly air dry. Finally, a cover slip was applied to slides using a low fluorescence glycerol mounting media and read with a fluorescent microscope at 40X oil objective. The detailed FAT protocol is available at http://www.cdc.gov/rabies/pdf/RabiesDFASPv2.pdf.

Because the humidity chamber used was in a common use area and no step during FAT inactivates rabies virus, microscope slides with tissue sections were exposed to ultraviolet light for 30 minutes prior to the beginning of the test. This was not necessary with DRIT because the initial step, immersion in 10% phosphate buffered formalin, inactivates the rabies virus.

Results

The rabies virus antigen was identified in all tissue types of naturally and experimentally infected Arctic foxes that were tested, except in testes (Table 2). The sample size for testes was relatively small, consisting of only five foxes. Spleen and stomach had the highest frequency of virus detection with 71.4% (10 of 14 foxes) and 60.0% (9 of 15 foxes), respectively, when tested with the FAT (Figure 5). However, pancreas had the highest ratio of virus detection with 15.4% (2 of 13 foxes) when tested with the DRIT (Figure 5). Liver was the only tissue that tested positive by DRIT, but was negative by FAT for all animals tested.

While no individual tissue type tested positive for rabies viral antigen in all foxes, using both spleen and stomach tissues to test for rabies infection would increase the chances of detection to 84.5% which is a higher rate of detection than using either alone. This is also true for using a combination of esophagus and spleen to detect rabies viral antigen or using lung and stomach (Table 3). These three combinations of tissues provide the best chances of detecting rabies virus antigen if brain tissue is unavailable for rabies diagnosis.

Using McNemar's test, significant differences in detection rates between the two methods were found in spleen and stomach ($p = 0.0156$ and $p = 0.0117$, respectively) with $\alpha=0.05$ considered significant. For these two tissue types the null hypothesis, that both testing methods have the same rate of detection, was rejected. Standard errors were calculated according to the binomial probability distribution (Figure 5).

Several tissue sections were cut at 8um, 4um, and 2um, to determine if thinner sections improved clarity and visibility of rabies viral antigen detection using the DRIT and FAT. However, results were the same regardless of the tissue thickness (data not shown).

In efforts to better understand the differences in detection between FAT and DRIT in different tissue types, specifically in spleen and stomach which had the highest percentage of rabies virus antigen in FAT, I mixed rabies positive Arctic fox brain tissue with both spleen and stomach using mortar and pestle. Tissues were frozen in O.C.T. compound and sections were cut into 8um sections onto microscope slides, as was performed for all other tissue samples. I carried out DRIT on these slides, as well as FAT, to determine if enzymatic inhibition occurred resulting in a false negative in these tissues. Results for these slides were positive, indicating that enzymatic inhibition did not occur.

Discussion

Rabies virus antigen was detected in organ tissues of naturally and experimentally infected Arctic foxes. Significant differences between the two testing methods were

observed in spleen and stomach tissues using McNemar's test. Experimentally infected foxes followed a trend of longer incubation periods resulting in a greater number of tissues testing positive for rabies virus antigen (Table 1 and 2). Spleen and stomach tissues are the best substitute for non-neuronal tissues if non-neuronal tissues are unavailable.

FAT and DRIT

The results demonstrate that FAT detected rabies viral antigen in Arctic fox tissues more frequently than the DRIT, which was only developed and optimized for use in neuronal tissue. Optimization of this test in non-neuronal tissues may include the omission of a counterstain or immediate preservation of fresh tissue samples in liquid nitrogen. Tissues that are exposed to freeze thaw cycles, or are not immediately preserved in liquid nitrogen may fracture which may negatively impact results of immunohistochemical tests (Renshaw 2007). Therefore, using fresh tissue samples from foxes and immediately immersing tissues in liquid nitrogen would better preserve tissue structure.

The failure of FAT to detect rabies virus antigen in liver may indicate that the sensitivity of the FAT in Arctic foxes tissues needs to be further examined. Newer equipment to ensure processing of consecutive tissue slices would help to confirm results, as would an increase in the number of tissue samples taken per organ.

Although reverse transcriptase polymerase chain reaction (RT-PCR) can be a highly useful molecular method for rabies virus amplification, this method was not

chosen for this study. Some tissue samples were only available in limited quantities and no excess tissue was available after performing the DRIT and FAT. Furthermore, for some lyssaviruses, the antigen epitope may be more highly conserved than the nucleotide sequence, so testing by molecular methods, such as RT-PCR, may be less sensitive than FAT (Trimarchi and Nadin-Davis 2007). However, in circumstances where tissues are decomposed the use of a molecular method may be more sensitive than FAT (Trimarchi and Nadin-Davis 2007), but this is questionable as RNA may be degraded consequently preventing detection. However, due to the discrepancies observed in the DRIT and FAT results, this method is being reconsidered for a continued investigation of this study and results will then be compared between the three methods.

Non-neuronal Tissues

This study tested whether the DRIT was suitable for use in non-neuronal tissues if brains are unavailable. It may be highly useful to find a substitute for brain tissue in circumstances where brain tissue is not available, e.g., when an animal is shot in the head. It is not uncommon for animals that are suspect rabid to be killed by a head-shot, which makes rabies testing at State Virology Labs unreliable or not possible. In cases where the animal cannot conclusively be determined negative for rabies virus and there has been human exposure, the exposed person is recommended to undergo rabies post-exposure prophylaxis treatment (PEP). Approximately 40,000 people receive PEP for rabies exposure each year in the United States (Meltzer and

Rupprecht 1998), with an estimated cost of $2000-4000 per person (Dhankhar et al. 2008). In addition, while rabies PEP is considered safe, side effects are common and can be moderate to severe (Mattner et al., 2007). Therefore, if another tissue could be used to assess rabies infection in situations when brain tissue is unavailable, then thousands of dollars could potentially be saved annually by avoiding unnecessary PEP treatment costs. This may also be useful in countries with high rates of human exposure to rabies where PEP is not only costly but in limited supply.

A study by Debbie and Trimarchi (1970) found no virus in spleen, liver, or ovary of red foxes, contrasting greatly with this study where spleen had the highest viral antigen detection using FAT. Numerous factors may be responsible for the differences in results between the two studies. Perhaps red foxes and Arctic foxes sequester the virus in organs differently, or there may have been differences in duration of infection or age. Although the sex of each fox was known, age, duration of infection, or whether the animal was symptomatic was unknown in this investigation and the study by Debbie and Trimarchi (1970). Furthermore, the study by Debbie and Trimarchi (1970) had the highest rates of rabies viral detection in esophageal tissue, adrenal gland, and salivary gland, 100%, 92% and 92%, respectively. I detected rabies viral antigen in only 26.7% of esophageal tissue tested by FAT and salivary glands were not tested as heads were unavailable due to prior FAT testing of the brains. Adrenal gland was not included as I focused on organs that are most easily accessible to scavenging foxes. However, it would be very useful to test adrenal and salivary gland tissues in a future study to determine if detection rates

in Arctic foxes are as high as in red foxes. If so, then these tissues may be the best alternative when brain is unavailable.

Effects of Sex

Rabies in Arctic foxes predominately affects young males (Crandell 1991, Ballard et al. 2001). This is further supported by a study by Kantorovich (1964) who demonstrated that males and young Arctic foxes were twice as likely to have rabies virus isolated from brain tissue than female foxes. In this study, all male foxes had rabies viral antigen detected by FAT in three or more tissues, with fox 9703, a male, having the most tissues infected (Table 2). In contrast, detection of rabies viral antigen in female foxes ranged from zero tissues infected, fox 94043, to five tissues infected, fox B100 and fox B200, as detected by FAT (Table 2).

Experimental Foxes

Of the experimental animals, fox EXP10 was challenged with 500,000 MLD_{50} and had the shortest incubation period of 13 days. Fox EXP12, also challenged with 500,000 MLD_{50}, had the longest incubation period of 20 days. Fox EXP349 had the lowest inoculation dose, 50,000 MLD_{50}, and would be expected to have a longer incubation period, but succumbed to rabies by day 18. Interestingly, the number of tissues identified as rabies-positive by FAT in the experimentally infected foxes corresponded to length of incubation period, so the longer the incubation period the more tissues were infected (Table 1 and 2). This is in agreement with studies that

have demonstrated that duration of incubation period increases the distribution of viral antigen within tissues (Murphy 1973, Fekadu and Shaddock 1984). Fox EXP12 had the longest incubation period and the greatest number of rabies positive tissues by FAT, and fox EXP10 had the shortest incubation period and the fewest rabies positive tissues by FAT (Table 2). Presumably, the longer incubation period allowed the virus to travel to more organs within the body. This could also indicate that incubation time is a source of variability in wild-caught arctic foxes and that stomach and spleen are among the organs to be infected first aside from neuronal tissues.

It is unknown how long the rabies virus can survive and remain infective in frozen carcasses. In human cadavers, the virus may remain infective for several weeks or months (Krauss 2003), so it is likely that the virus remains viable for an extended period of time in animal carcasses in the Arctic (Crandell 1991, Hanlon et al. 2007). It is, therefore, plausible that rabies virus remains infectious in Arctic fox carcasses and that scavenging animals may acquire the virus through ingestion or orally through abrasion of mucous membranes by bone shards and frozen tissue. A study to determine the infectivity of archived frozen tissue samples is recommended.

Rabies and Climate Change

A change in the Arctic climate and associated changes in sea ice extent and quality has a very real possibility, if not certainty, of shifting the spread of rabies within Arctic fox populations and of potentially spreading the virus into other marine and terrestrial wildlife populations. The loss of sea ice, as witnessed in 2007-2010

(Perovich et al. 2008, National Snow and Ice Data Center, 2010), forces ice-adapted marine species onto terrestrial haul-outs (e.g., walruses (*Odobenus rosmarus divergens*) and seals; Jay and Fischbach 2008) and could lead to increased interactions of these species with typical rabies hosts such as Arctic foxes (Burek et al. 2008). This may increase exposure of subsistence hunters, as handling or ingestion of raw infected meat may transmit the rabies virus (Wallerstein 1999).

Conclusions

To my knowledge, this is the first study to examine rabies distribution within soft tissues of naturally and experimentally infected Arctic foxes, using both FAT and DRIT. Fluorescent antibody testing detected rabies viral antigen in a greater number of non-neuronal tissues than the DRIT. Further studies are needed to optimize detection of rabies viral antigen by DRIT in non-neuronal tissues. Comparison of DRIT and FAT to RT-PCR is also recommended. I found stomach and spleen to be the best substitutes for neuronal tissue if neuronal tissue is unavailable. Experimental studies of rabies infection in Arctic foxes would yield invaluable information about duration of infectivity, potential correlation of incubation period with viral antigen spread to other non-neuronal tissues, and viral excretion. Further studies are recommended to assess the persistence of infectivity of rabies virus in frozen Arctic fox tissues, and to assess transmission of rabies virus by ingestion.

Acknowledgements

I am grateful to Erich Follmann for his unending patience, guidance, humor and generosity. He taught me much and I will miss him deeply. To my husband, who provided unwavering support, I could not be more thankful. To my committee members, thank you so much for your time, guidance, and advice. This project would not have been possible without help from Alaska State Virology Laboratory, Center for Disease Control, U. S. Department of Agriculture APHIS, Don Ritter, Mike Harris, Barbara Taylor, and Mike Niezgoda. Thank you for the use of your facilities, equipment and time. This project was supported by grant RR016466 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH); Alaska EPSCoR funded by National Science Foundation (NSF) award 0701898 and the State of Alaska.

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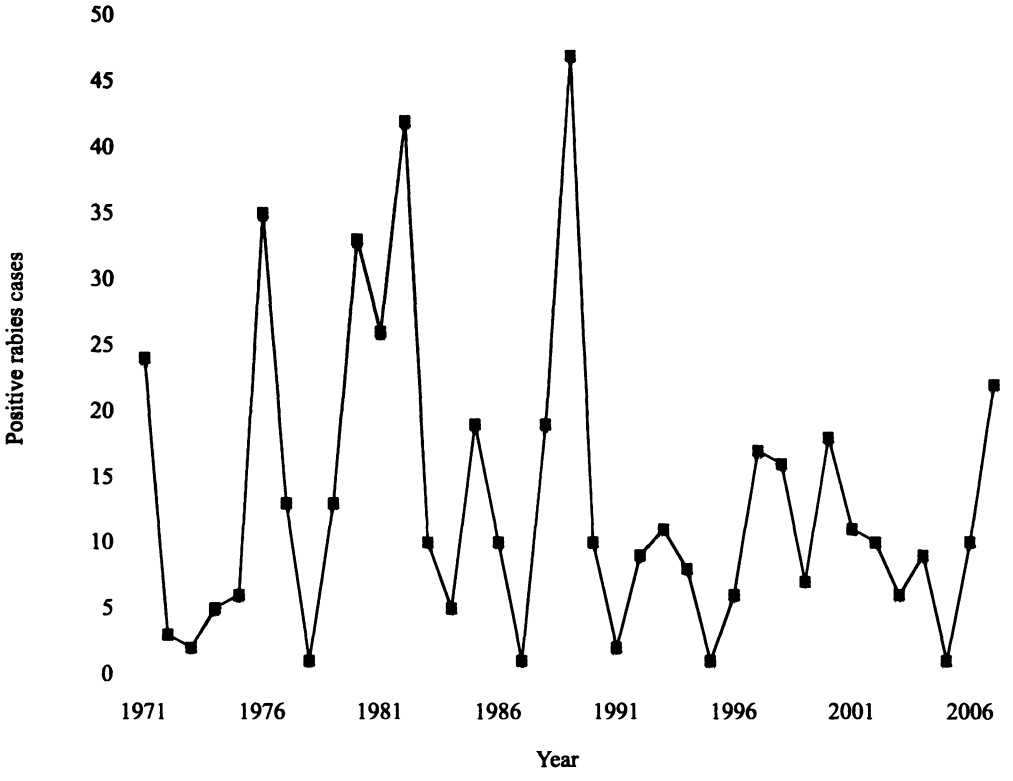

Figure 1. Epizootics in Arctic foxes in Alaska 1971-2007. Data provided by Don Ritter. Used with permission.

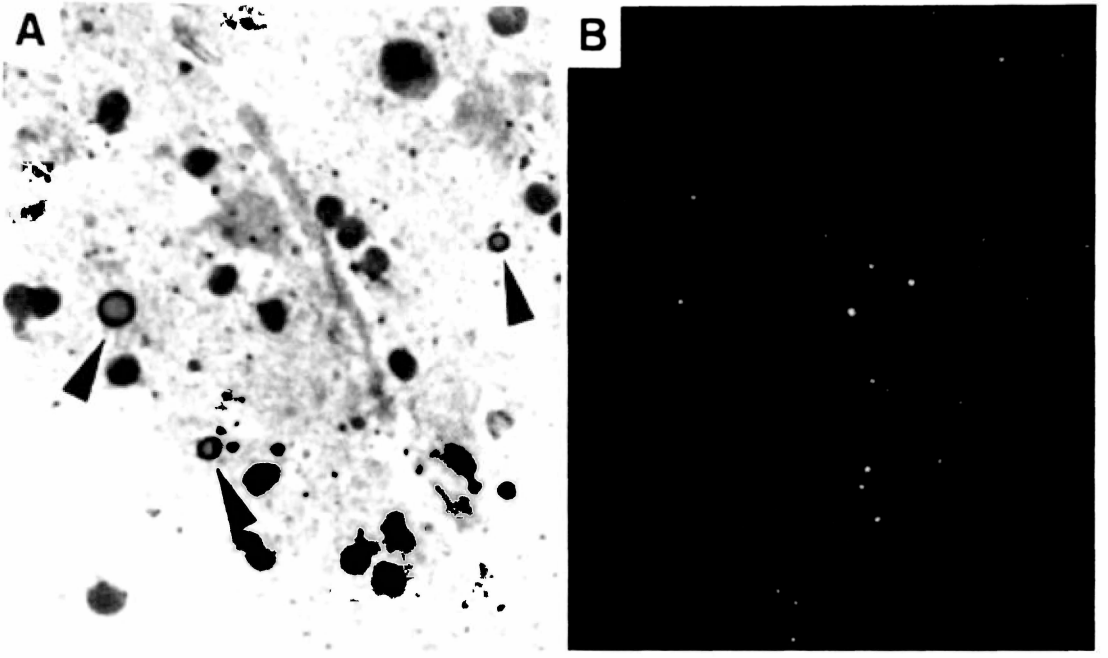

Figure 2. Rabies virus antigen as detected by DRIT and FAT. Staining of rabies virus antigen (arrows) with direct rapid immunohistochemistry test (DRIT) (A) and staining of rabies virus antigen (green) with fluorescent antibody test (FAT) (B). Photograph courtesy of Mike Niezgoda. Used with permission.

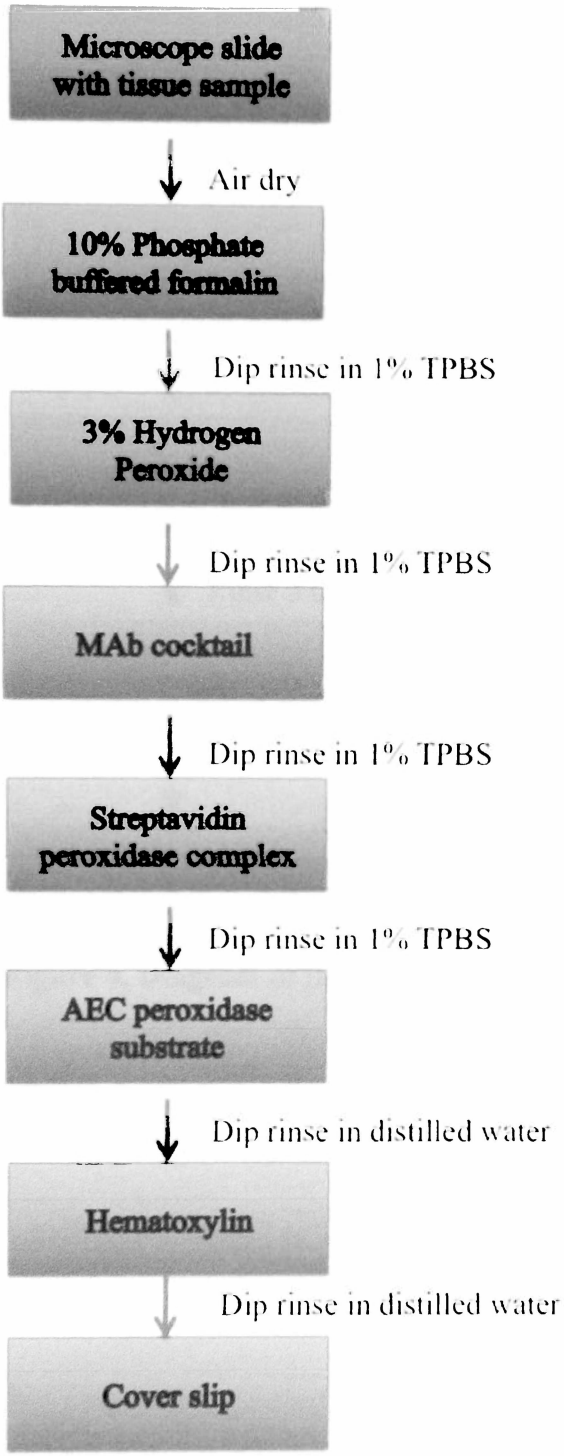

Figure 3. Diagram of direct rapid immunohistochemistry test (DRIT).

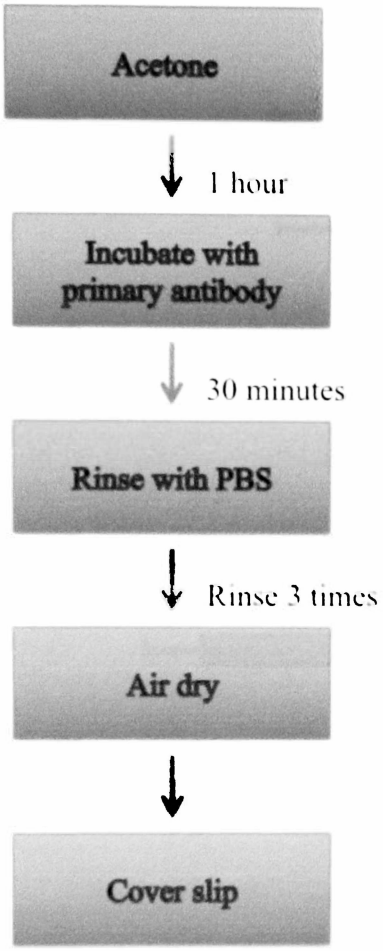

Figure 4. Diagram of fluorescent antibody test (FAT).

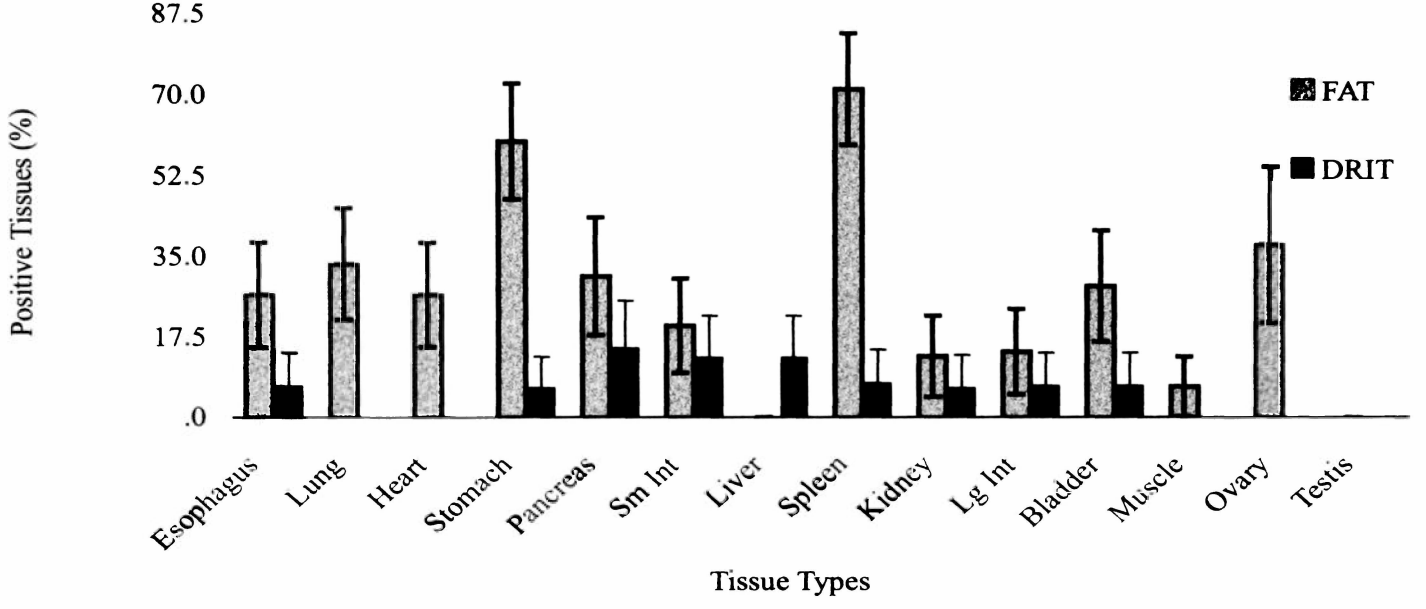

Figure 5. Percentage of Arctic fox tissues positive for rabies virus antigen. Percentage of non-neuronal organ tissues from naturally and experimentally infected Arctic fox that tested positive for rabies virus antigen using the direct rapid immunohistochemistry test (DRIT) (black bars) and fluorescent antibody test (FAT) (gray bars). Standard error bars shown.

Table 1. Rabies infected Arctic fox identification and information

Fox ID	Date Received by ASVL	Necropsy Date	Infection Status	Sex	Incubation Period (days)
EXP10	N/A	May 11, 2009	Experimentally infected	Male	13
EXP12	N/A	May 20, 2009	Experimentally infected	Female	20
EXP349	N/A	Mar 31, 2007	Experimentally infected	Female	18
B100	N/A	Apr 14, 2007	Naturally infected	Female	N/A
B200	N/A	Apr 14, 2007	Naturally infected	Female	N/A
B300	1994	Apr 14, 2007	Naturally infected	Female	N/A
B400	N/A	Apr 14, 2007	Naturally infected	Male	N/A
94041	1994	Apr 20, 2007	Naturally infected	Female	N/A
94034	1994	Apr 28, 2007	Naturally infected	Female	N/A
94038	1994	Apr 20, 2007	Naturally infected	Male	N/A
94043	1994	Apr 20, 2007	Naturally infected	Female	N/A
94046	1994	Apr 20, 2007	Naturally infected	Female	N/A
9703	1997	Mar 31, 2007	Naturally infected	Male	N/A
9705	1997	Mar 31, 2007	Naturally infected	Female	N/A
9709	1997	Mar 31, 2007	Naturally infected	Male	N/A

Table 2. Results for DRIT and FAT. Naturally and experimentally infected Arctic fox tissues that tested positive for rabies virus antige direct rapid immunohistochemistry test (DRIT) and fluorescent antibody test (FAT). A cell containing +/- or -/+, the FAT result is given then the DRIT result. A single entry indicates the same result for both tests. NA = tissue unavailable due to decomposition.

	Negative Control	94043	94038	B100	B200	B300	B400	94041	9705	9709	94034	9703	94046	EXP 349	EXP 10	EXP 12
Esophagus	-	-	-	+/-	-/+	+/-	+/-	-	-	-	-	+/-	-	-	-	-
Lung	-	-	-	-	-	+/-	+/-	+/-	-	-	-	+/-	-	+/-	-	-
Heart	-	-	-	+/-	+/-	-	-	-	-	+/-	+/-	-	-	-	-	-
Stomach	-	+	+/-	+/-	-	-	+/-	+/-	-	+/-	+/-	-	+/-	-	-	+
Pancreas	-	NA	+/-	+	+/-	-	-	-	-	-	-/+	-	NA	-	-	+/-
Small Intestine	-	-	-	+/-	-	-	-	-	-/+	-	-	-	-	-	+/-	+
Liver	-	-	-	-	-	-	-/+	-	-	-	-/+	-	-	-	-	-
Spleen	-	NA	+/-	-/+	+/-	-	-	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Kidney	-	-	-	-	-	-	-	-	-	-/+	-	+/-	-	+/-	-	-
Large Intestine	-	-	-	-	+/-	-	-	-	-	-/+	-	+/-	NA	-	-	-
Bladder	-	-	+/-	-	-	-	-	-	-	-	-	+/-	NA	-	+/-	+
Muscle	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-
Ovary	-	NA		-	+/-	-		-	+/-		-/NA		NA	+/NA		-
Testis	-		-				-			-		-			-	

Table 3. Combination of non-neuronal naturally infected Arctic fox tissues. Combination of tissues that provide highest detection rates of rabies viral antigen by fluorescent antibody test (FAT).

Tissue Combination	Percentage of detection	Tissue Combination	Percentage of detection
Esophagus and spleen	84.6	Esophagus and large intestine	38.5
Lung and stomach	84.6	Esophagus and bladder	38.5
Stomach and spleen	84.6	Esophagus and muscle	38.5
Esophagus and stomach	76.9	Lung and liver	38.5
Lung and spleen	76.9	Lung and kidney	38.5
Stomach and kidney	76.9	Heart and pancreas	38.5
Stomach and large intestine	76.9	Heart and large intestine	38.5
Lung and heart	69.2	Heart and muscle	38.5
Heart and stomach	69.2	Pancreas and kidney	38.5
Heart and spleen	69.2	Esophagus and small intestine	30.8
Stomach and pancreas	69.2	Esophagus and liver	30.8
Stomach and bladder	69.2	Heart and small intestine	30.8
Stomach and muscle	69.2	Heart and liver	30.8
Pancreas and spleen	69.2	Pancreas and large intestine	30.8
Small Intestine and spleen	69.2	Pancreas and bladder	30.8
Spleen and muscle	69.2	Pancreas and muscle	30.8
Lung and pancreas	61.5	Pancreas and small intestine	23.1
Stomach and small intestine	61.5	Pancreas and liver	23.1
Stomach and liver	61.5	Small Intestine and kidney	23.1
Liver and spleen	61.5	Small Intestine and large intestine	23.1
Spleen and kidney	61.5	Small Intestine and bladder	23.1
Spleen and large intestine	61.5	Kidney and large intestine	23.1
Spleen and bladder	61.5	Kidney and bladder	23.1
Esophagus and heart	53.8	Kidney and muscle	23.1
Esophagus and lung	46.2	Large Intestine and bladder	23.1
Lung and large intestine	46.2	Large Intestine and muscle	23.1
Lung and bladder	46.2	Bladder and muscle	23.1
Lung and muscle	46.2	Small Intestine and muscle	15.4
Heart and kidney	46.2	Liver and kidney	15.4
Heart and bladder	46.2	Liver and large intestine	15.4
Esophagus and pancreas	46.1	Liver and bladder	15.4
Lung and small intestine	46.1	Small Intestine and liver	7.7
Esophagus and kidney	38.5	Liver and muscle	7.7

General Conclusions

This study supports my hypothesis by demonstrating that rabies virus antigen is present in the organ tissues of naturally and experimentally infected Arctic foxes. Because rabies virus antigen was detected in various tissues regardless of length of freezer storage, it is plausible that the virus remains infectious in fox carcasses during the winter months on the Alaskan tundra and elsewhere in the Arctic. If the virus remains infectious, then scavenging foxes may acquire the disease by feeding on infected carcasses (i.e. soft tissues), resulting in the propagation of the virus and the spread of the disease within a fox population. Spleen and stomach tissues had the highest rates of rabies antigen virus detection and are the best substitute for non-neuronal tissues if non-neuronal tissues are unavailable. Furthermore, stomach and spleen tissues may provide a useful tool for surveillance studies if brain tissue is unavailable.

The habitat of Arctic foxes may shift or decrease, which may impact their relationship with red foxes. Traditionally red foxes reside inland and adjacent to Arctic foxes, but encroachment by the red fox is already occurring in the Arctic in some areas (Pamperin et al. 2006, Fugeli and Ims 2008). In Scandinavia this interaction with red foxes has been observed and is leading to rapid declines of the already endangered Arctic foxes population in this area (Selås and Vik 2006). This may result in an increased numbers of rabies cases as contact between these species increases (Pamperin et al. 2006, 2008). Rabies outbreaks occur primarily along the

coast of northern and western Alaska, because rabies is enzootic in fox populations in these areas (State of Alaska Epidemiology Bulletin No. 1 2002, No. 9 2004). However, interior Alaska could also be negatively affected by these climate changes, should rabies epizootics enter this area from the north and/or west due to increased transmission of the rabies virus between red foxes in overlap zones and those in the interior.

Several oral bait vaccines have been successfully tested in Arctic foxes (Follmann et al. 1988, 1992). However, given the prolific nature of these canids, their winter movement onto the ice pack, and the high rate of rabies infection within populations, it would be extremely difficult, if not impossible, to vaccinate enough animals to decrease the probability of an epizootic occurring. A high proportion of the fox population would need to be vaccinated, approximately 50-70% to be effective (Wandeler 1991) and to produce a reduction in epizootic frequency, perhaps even higher than what is feasible with a vaccination program. However, vaccination control could be accomplished where a resident population of arctic foxes that does not move widely occurs, a situation that is the case in the oil field of northern Alaska or in the Scandinavian Arctic where these foxes are critically endangered (Dalen et al. 2006, Pamperin et al. 2008). Difficulties with vaccinating wild animal populations emphasize the need of projects, such as this one, which seek to gain a better understanding of virus transmission and distribution.

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Appendix A

Niezgoda, Michael (CDC/OID/NCZVED) <man6@cdc.gov>
to Lori Gildehaus <lagildehaus@alaska.edu>
date Mon, Oct 4, 2010 at 6:23 AM
subject RE: Photograph comparing DRIT and DFA

Yes.... Please feel free to use the photos.

Don Ritter <donritter@gci.net>
to Lori Gildehaus <lagildehaus@alaska.edu>
date Fri, Sep 24, 2010 at 12:52 PM
subject Cyclic Rabies
The file Cyclic Rabies is in the attached file

Appendix B

August 10, 2006

To: Erich Follmann, PhD
Principal Investigator

From: Bridget Stockdale, Research Integrity Administrator
Office of Research Integrity

Re: LBC Registration Application

Thank you for submitting an infectious agent registration for your upcoming research involving rabies virus. The University of Alaska Fairbanks Laboratory & Biosafety Committee has reviewed and approved the LBC registration beiow.

Protocol#: 06-03

Title: *Rabies virus in arctic fox (alopex lagopus): a pantropic study*

Received: August 4, 2006

Approved: August 10, 2006

Review Due: August 10, 2007

Please keep this registration current by informing the LBC (fycomp@uaf.edu) of any changes in location, personnel or methodology. Should an exposure occur, please complete and submit a UAF Accident/Incident Report (http://www.uaf.edu/safety/incidentreport.pdf).